IN THE U.S. PATENT & TRADEMARK OFFICE

Applicants:	Shozo KOYAMA et al.		
Serial No.:	10/786,369	Group:	1641
Filed:	Feb 26, 2004	Examiner:	Shafiqul Haq
For:	METHOD FOR PRODUCING AN ANTIGENIC SUBSTANCE AND ANTIBODY		

DECLARATION UNDER 37 C.F.R. § 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C., 20231

Sir:

I, Shozo KOYAMA, a nation of Japan, residing at 48-2, Oazasatoyamabe, Matsumoto-shi, Nagano 390-02, Japan, do hereby declare as follows:

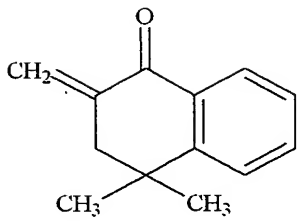
I am a co-applicant of the invention as described and claimed in the specification of the above-identified application.

I am familiar with the Final Office Action dated October 19, 2007, in which claims 35 and 37 are rejected as being indefinite and as failing to comply with the written description requirement.

To show that the compound of Formula 3-a which is different from Yoshixol is surely effective for preventing and/or treating cancer, I carried out the experiments described below.

Experiments

The polycyclic compound of Formula 3-a, 4,4-dimethyl-2-methylene-3,4-dihydronaphthalen-1-one (hereinbelow referred to as "Yoshixol-7001"), was synthesized and tested for its effect.



1. Synthesis of Yoshixol-7001

To a dried 100 mL round-bottom recovery flask (eggplant type flask), 4,4-dimethyltetralone (17.2 mmol, 3 g) and anhydrous THF (30 mL) were added, and the resulting mixture was cooled to -10°C under stirring in nitrogen atmosphere. LDA (lithium diisopropylamide) (17.2 mmol, 8.6 mL) was added dropwise thereto over 15 minutes. The resulting mixture was stirred at -10°C for 15 minutes and then at -78°C for 2 hours, thereby preparing enolate.

To another dried 100 mL round-bottom recovery flask, diiodomethane (93.6 mmol, 25 g) and anhydrous THF (20 mL) were added, and the resulting mixture was stirred at -10°C and then left it to stand for 10 minutes. To this diiodomethane solution, the above-obtained enolate prepared in LDA was added dropwise slowly over 10 minutes using syringe. The resulting mixture was stirred for 2 hours, then heated gradually to room temperature and stirred for 12 hours. Solvent and diiodomethane was removed under reduced pressure to obtain 2-iodemethyl-4-4-dimethyl-1-tetralone. Thereafter, 2-iodemethyl-4-4-dimethyl-1-tetralone (17.2 mmol) was reacted with 2 equivalent of DBU (5.2 g, 34.4 mmol) at 90°C for 45 minutes under nitrogen atmosphere. After cooling the mixture to room temperature, 100 mL of benzene was added thereto, and the precipitated amine salt was removed by filtration. The obtained benzene solution was treated with 5% hydrochloric acid (100 mL x 2) and washed with saturated aqueous sodium carbonate solution. Benzene layer was dried over anhydrous magnesium sulfate, and the dried layer was passed through a thin layer of silica gel, followed by concentration under reduced pressure. The residue was distilled off using a vaccum pump to obtain 1.06g (33%)

of the desired compound (Yoshixol-7001).

1H-NMR (CDCl3, TMS, 400MHz) δ 1.39(s, 6H, CH3), 2.74(s, 2H, CH2), 5.44(s, 1H, =CH), 6.30(s, 1H, =CH), 7.56-7.31(3H, Ar), 8.13(1H, Ar)

2. Preparation of vaccine and examination of its effect

(1) Vaccine against murine L1210 leukemia

(i) Preparation of vaccine

Mouse leukemia cells L-1210 (Cancer research institute, Tokyo) was incubated with 2 ml of culture medium (Minimum Essential Medium, Gibco Co.; glutamine, Dai-Nipon Pharmace. Co.) supplemented with fetal bovine serum (Dai-Nipon Pharmace. Co.) under 5% CO_2 in an incubator at 37°C for 30 hours. When the number of cell reached about 1 x 10^6, cell death is induced by adding 4 μl of 2 M Yoshixol-7001 solution in ethanol. Cell viability was determined by an uptake of methylene blue. After confirming extinction of cells in culture medium, the culture medium cotaining extincted cells was centrifuged at 1,000 rpm for 5 minutes and the supernatant was removed by aspiration. To the sediment, 0.9 cc of physiological saline was added, and the mixture was stirred and centrifuged at 1,000 rpm for 5 minutes, followed by removing the supernatant by aspiration. This procedure was repeated twice. To the rinsed sediment, 0.9 cc of physiological saline was added, and the resulting mixture was stirred, followed by filtrating the mixutre through Millipore-filter of cellulose acetate having 0.45 μm pore to obtain a vaccine against leukemia.

(ii) Effect of vaccine

The filtered solution obtained in (1) (i) (i.e., a vaccine against leukemia) was injected intraperitoneally into CDF-1 strain of male mice (Charles-River Co, Saitama) in an amount of 0.2 cc per a mouse (vaccination). Thirty one days after the vaccination, 0.3 cc of culture medium of mouse leukemia cells L-1210 (1 x 10^6

cells/mL) which had been cultured independently from the above-described culture was intraperitoneally injected to vaccinated mice and control mice (without vaccination), respectively. A difference of timing of death due to progression of leukemia between vaccinated mice and control mice was investigated. The result are shown in Figure 1.

As shown in Figure 1, all control mice died within 9 days after the transplantation of leukemia cells. In contrast, vaccinated mice began to die 13 days after the transplantation, and the last mouse survived for as long as 19 days.

(2) Vaccine against murine B16 melanoma

(i) Preparation of vaccine

Mouse melanoma cells (B16; purchased from Riken Cell Bank) were incubated in 2 ml of culture medium (Minimum Essential Medium, Gibco Co.; glutamine, Dai-Nipon Pharmace. Co.) supplemented with fetal bovine serum (Dai-Nipon Pharmace. Co.) under 5% CO_2 in an incubator at 37°C for 30 hours. When the number of cells reached about 2 x 10^5, cell death was induced by adding 4 μl of 2 M Yoshixol-7001 solution in ethanol. After confirming extinction of cells in culture medium, the culture medium containing extincted cells was centrifuged at 1,000 rpm for 5 minutes and the supernatant was removed by aspiration. To the sediment, 0.9 cc of physiological saline was added, and the mixture was stirred and centrifuged at 1,000 rpm for 5 minutes, followed by removing the supernatant by aspiration. This procedure was repeated twice. To the rinsed sediment, 0.9 cc of physiological saline was added, and the resulting mixture was stirred, followed by filtrating the mixutre through Millipore-filter of cellulose acetate having 0.45 μm pore to obtain a vaccine against melanoma.

(ii) Effect of vaccine

The filtered solution obtained in (2) (i) (i.e., a vaccine against melanoma) was

injected intraperitoneally into C57/BL mice (female, 6 weeks old, purchased from Nihon SLC Co.) in an amount of 0.2 cc per a mouse. Thirty days after the vaccination, 0.3 cc of culture medium of mouse melanoma cells (2×10^5/ml) which had been cultured independently from the above-described culture was transplanted to the back of vaccinated mice and control mice (without vaccination), respectively. The growth of melanoma cells B16 in each mouse was measured 45 days after the transplantation. The result are shown in Figure 2.

As shown in Figure 2 (top), the average amount of tumor in control mice reached more than 15 g. In contrast, an average amount of tumor in vaccinated mice was less than 2 g.

Figure 2 (bottom) shows the appearance of vaccinated mouse and control mouse (45 days after the transplantation).

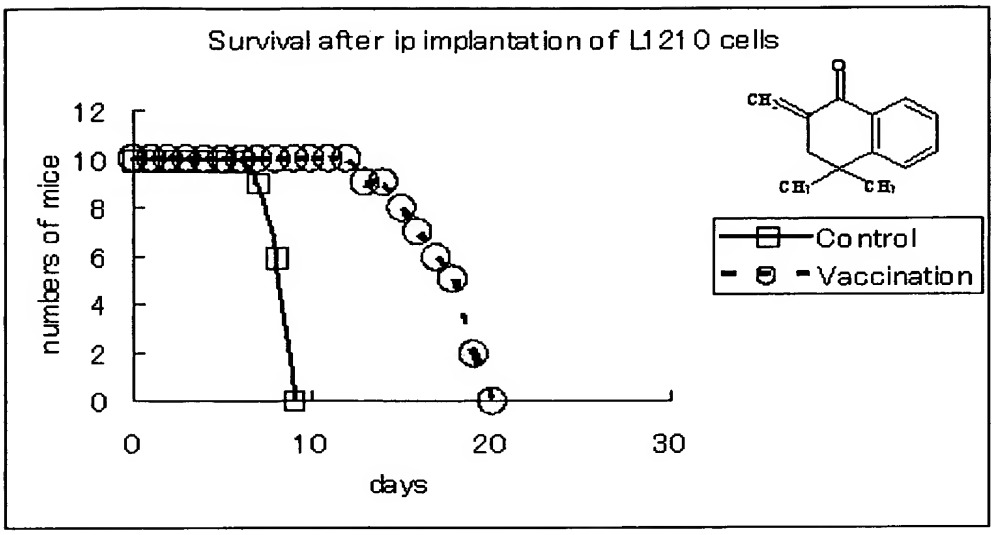
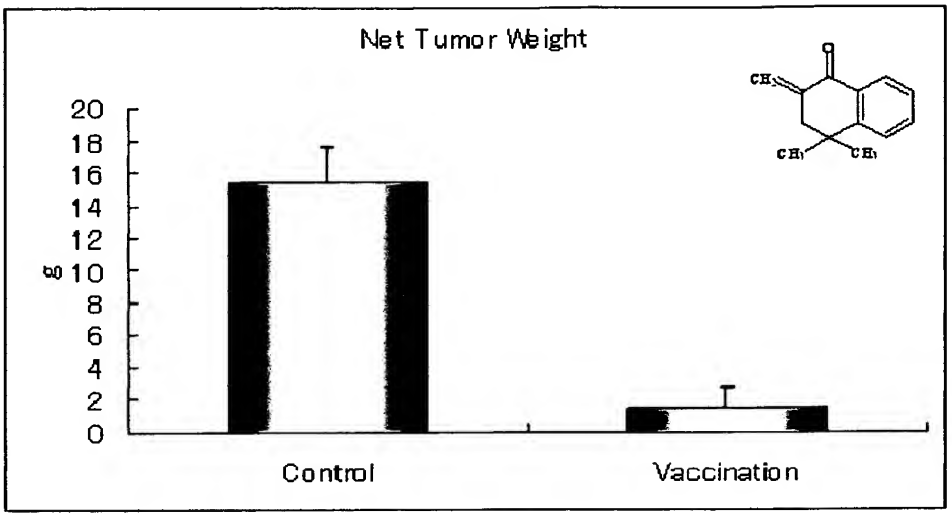

Survival after ip implantation of L1210 cells
numbers of mice
12
10
8
6
4
2
0
0
10
20
30
days
CH2
CH3
CH3
Control
Vaccination

Figure 1


Net Tumor Weight
20
18
16
14
12
10
8
6
4
2
0
Control
Vaccination

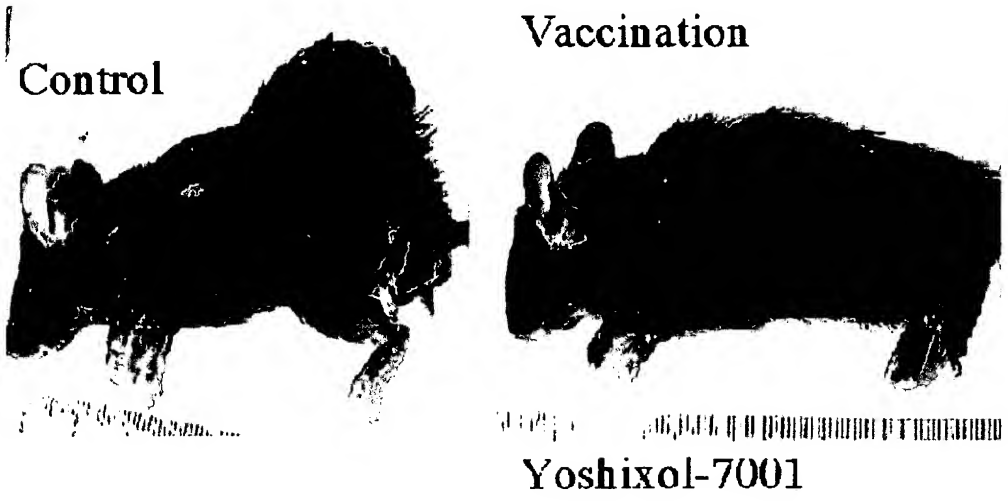

Control
Vaccination
Yoshixol-7001

Figure 2

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

This 11th day of June, 2008

Shozo KOYAMA